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(57) Abstract

The present invention relates to a real–time Polymerase Chain Reaction (PCR) method for the detection and quantification of variants of nucleic acid sequences which differ in the probe–bindung site. The method is based on the complete or partial amplification of the same region of the variants and the addition of two or more oligonucleotide probes to the same PCR mixture, each probe being specific for the probe–binding site of at least one variant. The method can be applied e.g. to estimate the viral load in a sample, to differentiate between subgroups, subtypes isolates or clades of a viral species or to estimate the impact of the viral load on tumorigenesis.

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Multiplex Real-Time PCR

The present invention relates to a real-time Polymerase Chain Reaction (PCR) method for the detection and quantification of variants of nucleic acid sequences which differ in the probe-binding site. The method is based on the complete or partial amplification of the same region of the variants and the addition of two or more oligonucleotide probes to the same PCR mixture, each probe being specific for the probe-binding site of at least one variant. The method can be applied e.g. to estimate the viral load in a sample, to differentiate between subgroups, subtypes, isolates or clades of a viral species or to estimate the impact of the viral load on tumorigenesis.

Background of the invention

The detection and quantification of nucleic acid sequences is of importance for a wide range of experiments and applications. Several methods for the detection and quantification of nucleic acid sequences have been described previously. Most methods are based on polymerase chain reaction (PCR): The PCR is used to amplify a segment of DNA flanked by stretches of known sequences. Two oligonucleotides binding to these known flanking sequences are used as primers for a series of *in vitro* reactions that are catalyzed by a DNA polymerase. Typically, these oligonucleotides have different sequences and are complementary to sequences that (1) lie on opposite strands of the template DNA and (2) flank the segment of DNA that is to be amplified. The template DNA is first denatured by heat in the presence of large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences. Afterwards, the annealed primers are extended by the DNA polymerase. The cycle of denaturation, annealing, and DNA-synthesis is then repeated about 10 to 50 times. Since the products of one cycle are used as a

template for the next cycle the amount of the amplified DNA fragment is theoretically doubled with each cycle resulting in a PCR-efficiency of 100%.

The specific amplification of a target sequence is due to the annealing of the primers to a complementary region of the DNA. If the primer differs in its sequence from the sequence of the annealing region of the target DNA, the PCR may fail. Accordingly, if a target sequence is analyzed that differs between samples in the primer-annealing region, the amplification of the target sequence in some samples will fail or will be less efficient. Therefore, degenerated primers are often used, i.e. primers that have unspecific nucleotide analogous at the positions at which the sequence varies between samples.

If two or more target sequences are amplified simultaneously in the same PCR reaction, a multiplex PCR is performed. Then, more than one primer pair is added to the PCR mixture and each primer pair allows the specific amplification of one target sequence.

The enzyme used for PCR is specific for DNA. If an RNA template is amplified by PCR, the RNA has first to be transcribed into complementary DNA (cDNA) by the enzyme reverse transcriptase. Afterwards the cDNA is used as a template in a PCR. Accordingly, the method of the amplification of RNA is called reverse-transcriptase (RT) PCR.

The PCR results in a large copy number of the sequence flanked by the primers. The large copy number of this sequence allows the detection and quantification of the target sequence after the PCR reaction. The detection of the amplification products is usually performed by gel electrophoresis and staining of the DNA. The intensity of the band after gel electrophoresis also allows to estimate the copy number of the

sequence of interest in the original sample mostly by comparison with a standard with a known copy number (*Sambrook et al., Molecular Cloning, 2nd edition, cold spring harbor laboratory press 1989, p. 14.30).*

The conventional PCR is widely used. However, the method has several disadvantages that are mostly linked with the detection of the amplification products by gel electrophoresis. The gel electrophoresis requires additional handling of the sample which is time-consuming and prone to sample mix-ups. In addition, the sensitivity of the detection method is low. Finally, quantification of the copy number of the template sequence requires a standard and is often difficult.

More recently, a new technology for the detection and quantification of target sequences was developed which does not show the disadvantages mentioned above. The method is called "real-time" PCR. Here, the DNA generated within a PCR is detected on a cycle-by-cycle basis during the PCR reaction. The amount of DNA increases the faster the more template sequences are present in the original sample. When enough amplification products are made a threshold is reached at which the PCR products are detected. Hence, amplification and detection are performed simultaneously in the same tube.

Most instruments that are used for a real-time PCR detect an increase of fluorescence of a specific wave length as a result of an increasing amount of PCR product. For example, the Applied Biosystems Prism 7700 sequence detection system is based on the combination of PCR and hybridization of a fluorogenic, target-specific probe. The probe is an oligonucleotide with both a reporter and a quencher dye attached at the 5´and 3´end respectively. The fluorescence of the reporter dye is efficiently quenched by the quencher dye as long as both fluorochromes are present in close proximity. If the target sequence is present, the probe anneals between the forward

and reverse primers. During PCR amplification and thus elongation of the primers the probe is cleaved by the 5'nuclease activity of the DNA-polymerase. This cleavage of the probe separates the reporter dye from the quencher dye, making reporter dye signal detectable. Additional reporter dye molecules are cleaved from their respective probes with each cycle, effecting a proportional increase in fluorescence intensity of the reporter dye and a decrease of the fluorescence intensity of the quencher dye. An algorithm of the software of the instrument compares the amount of reporter dye emission with the quenching dye emission once every few seconds during the PCR reaction, generating a normalized reporter signal ΔR_n. The first cycle in which the normalized reporter signal is above a defined threshold is defined as the threshold cycle C_T. The C_T value is proportional to the copy number of the template and used for quantification *(Heid, et al., Genome Research, 6: 986ff)*. The real-time PCR provides greater quantitative precision and dynamic range compared to other quantitative PCR methods, and is easier to handle.

If the template is not DNA but RNA a real-time reverse-transcription (RT) PCR is performed. As described for the conventional PCR the RNA is first transcribed into cDNA before the actual real-time PCR is performed.

Detailed description of the invention

For the determination of the viral load in samples from animals infected with the same type of virus a real-time PCR was performed. After detection and quantification of the viral nucleic acid sequence by real-time PCR the viral load was calculated. Considerable variation was found between the samples. In some samples the calculated copy number of the target sequence was very small or even no target sequence was detected. Unexpectedly, the calculated viral load did not correlate with

the severity of the disease of those animals the samples were taken from. In order to verify the results of the real-time PCR, a conventional PCR with staining of the amplified DNA after gel electrophoresis was performed. Although this method is much less sensitive as compared to the real-time PCR method, amplification products were surprisingly detected in all analyzed samples, i.e. even for samples in which no viral sequences were detected by real-time PCR, positive results were obtained by conventional PCR.

Further investigations showed that the animals, the samples were taken from, were infected with different subtypes of the same virus. The subtypes were characterized by variants of the viral nucleic acid sequence. Apparently, some variants of a nucleic acid sequence that were present in specific viral subtypes, were not detected by real-time PCR.

Accordingly, it was an object of the present invention, to provide a real-time PCR method for the detection and/or quantification of variants of a nucleic acid sequence.

The problem underlying the present invention is solved by the complete or partial amplification of the same region of variants of a nucleic acid sequence comprising nucleotide variations within the probe-binding site and the addition of two or more oligonucleotide probes to the same PCR mixture, each probe being specific for the probe-binding site of at least one variant. Said variants of a nucleic acid sequence are found e.g. in different subtypes of phylogenetically related groups of organisms such as in subtypes of families, genera, and species. The variants analyzed by a method according to the present invention are preferably derived from subtypes of a species such as e.g. clades, isolates or breeds. The variants of nucleic acid sequences may be identical in 50 to 70%, preferably 70 to 90% and most preferably 90 to 99% of the nucleotides.

The nucleic acid sequence of the different subtypes may differ not only in the probe-binding site but also in the primer-binding site. In this case, primers may not anneal to the primer-binding site, resulting in PCR failure. Hence, according to a preferred embodiment of the invention, more than one primer pair is added to the reaction mixture, wherein each primer specifically anneals to the nucleic acid sequence of at least one subtype (multiplex real-time PCR).

The primers and probes used for the method according to the present invention should be at least 60 to 80%, preferably 80 to 90%, and most preferably 90 to 100% homologous to the nucleic acid sequence of at least one variant of the nucleic acid sequence.

Within the completely or partially amplified region more than one probe-binding site may be included. If in this case the complete region is amplified the amplification products have more than one probe-binding site and more than one probe may anneal to the amplification product. This may e.g. cause interactions between the reporter and quencher dyes of the different annealed probes influencing the quantification. Hence, according to a preferred embodiment of the invention, two or more parts of the region of each variant are amplified, each part of the region comprising only one probe-binding site.

When two or more parts of the region of the nucleic acid sequence are amplified, primer pairs and probes may be chosen to be specific for one variant. In this case, the fluorescence signal of a specific probe may be characteristic for a specific variant.

The probes are labeled at the 3-prime end with a quencher and at the 5-prime end with a reporter dye. According to a preferred embodiment of the invention, different

probes are labeled with the same quencher dye but with different reporter dyes. In that case, the different amplification products can be distinguished. Any reporter dye can be attached to the probe. However, preferably FAM™ or VIC™ is used as a reporter dye.

The differentiation between amplification products using different reporter dyes may be applied for the classification of subtypes. First the inventors tried to classify the subtypes by monoplex real-time PCRs. In this case, only one primer pair and one probe is added to the reaction mixture, wherein the primer-pair and probe are chosen to be specific for one subtype. Then, PCR products should only be detected if this specific subtype is present in the sample. However, non-specific amplification and/or detection was observed in some cases, resulting in wrong classification of the subtype. Then the inventors used a multiplex real-time PCR according to the present invention for the classification. They added several of the subtype-specific primers and probes to a PCR mixture, wherein the different probes were labeled with different reporter dyes. In this case, a specific subtype is identified, when the fluorescence signal of the respective probe is detected. Using this multiplex real-time PCR method, all subtypes could be classified correctly. Hence, according to a preferred embodiment of the present invention, a multiplex real-time PCR with subtype-specific primers and probes is performed for the classification of subtypes, wherein the probes are labeled with different reporter dyes.

The present invention may be used to study viral diseases such as diseases caused by lentiviruses. Lentiviruses are associated with immunodeficiency and malignacies. The mechanisms involved in tumorigenesis are still not fully understood, but it is suspected that a correlation between tumorigenesis and the viral load exists. Cats infected with Feline Immunodeficiency Virus (FIV) represent a model for the role of the viral load in the pathogenesis of tumors, since cats infected with FIV develop

quite often tumors, especially lymphomas. Accordingly, in a preferred embodiment of the present invention, the real-time PCR is especially used for the detection and/or quantification of nucleic acid sequences of different subtypes of lentiviruses, especially of FIV.

Since lentiviruses are retroviruses, the nucleic acid sequence of the genome present in the viral particles consists of RNA. According to the life cycle of a retrovirus, the RNA genome is transcribed into DNA after the infection of a host cell. Then, the transcribed, retroviral DNA may be integrated into the genome of the host cell, forming the so-called provirus. If the already integrated viral genome shall be analyzed and accordingly amplified, no reverse transciption is necessary before the real-time PCR is performed. However, if the genome of the viral particles is analyzed, a reverse-transcription (RT) real-time PCR is performed.

FIV isolates comprising known and unknown variants of the viral nucleic acid sequence have been analyzed by FIV specific real-time PCR. Accordingly, the present invention also provides oligonucleotide probes as well as primer pairs for the detection and/or quantification of variants of nucleic acid sequences derived from FIV. Preferably probes and primer pairs according to Seq. ID No. 1 to 21 are provided. The primers and probe according to Seq. ID No. 1 to 3 are especially used for the detection and/or quantification of clade A of FIV, whereas oligonucleotides according to Seq. ID No. 7 to 9 are especifically used for the detection and/or quantification of clade B of FIV. Preferably, both sets of oligonucleotides are used simultaneously in a multiplex real-time PCR. Accordingly, with the two sets of oligonucleotides a method is provided which enables to distinguish between clade A and B of FIV in a sample. The probes according to Seq. ID No. 3, 6 or 9 may be combined with primers different from those according to Seq. ID No. 1, 2, 4, 5, 7 or 8, especially when FIV samples are analyzed that comprise unknown FIV isolates. In

one embodiment of the invention, the forward primer according to Seq. ID No. 1 is replaced by a primer according to Seq. ID No. 12. The probe according to Seq. ID No. 6 may be used in combination with the forward primers according to Seq. ID No. 4, 14 or 15 and the reverse primers according to Seq. ID No. 5 or 13. The probe according to Seq. ID No. 9 may be used in combination with the forward primer according to Seq. ID No. 7, 20 or 21 and the reverse primer according to Seq. ID No. 8. Furthermore, a set of oligonucleotides is provided comprising the probe according to Seq. ID No. 18, the forward primer according to Seq. ID No. 16, and the reverse primer according to Seq. ID No. 17 or 19.

The primers and probes with a sequence according to Seq. ID No. 1 to 21 as well as primers and probes with a homology of at least 70% to the sequences according to Seq. ID No. 1 to 21 may be used in general to amplify sequences specific for FIV. Additionally, the primer pairs can also be applied without the probes when a conventional PCR is performed instead of a real-time PCR.

In summary, the present invention provides a highly reliable and reproducible method for the detection and quantification of variable nucleic acid sequences.

Brief description of the tables

Table 1: The PCR-efficiencies of four different FIV isolates, and the corresponding sequences of the PCR-products are listed. The sequence given in this table is always from the same strand, despite the fact that the probe and the reverse primer bind to the complementary strand compared to the forward primer. The exact sequences (5'-3' orientation) of the primers and probe are described above.

Table 2: A comparison of the results from the real-time PCR and the sequence of the probe-binding site of PCR-products derived from four field isolates. The nucleotide sequence in the table is complementary to the sequence of the probe used. (nd) not determined.

Table 3: Results of the amplification of 30 unknown FIV isolates by the two monoplex real-time PCR assays 1010p and 1372p or by one multiplex real-time PCR composed of the 1010v- and the 1372p-assay. The subtype of the isolate was identified according to the results of the real-time PCR: (+) a PCR-product was detected with this assay; (-) no PCR-product was detected using this assay; (*) The subtype can only be determined by a multiplex real-time PCR.

Summary of the invention

A real-time Polymerase Chain Reaction (PCR) method for the detection and/or quantification of variants of a nucleic acid sequence, wherein the same region of said variants is completely or partially to be amplified, each variant differing in one or more nucleotides within the probe-binding site, said method comprising addition of two or more oligonucleotide probes to the same PCR mixture, each probe being specific for the probe-binding site of at least one variant.

The real-time PCR method as above, wherein said variants of the nucleic acid sequence differ in one or more nucleotides within the primer-binding sites and wherein more than one primer pair is added to the reaction mixture each primer specifically annealing to the primer-binding site of at least one subtype

The real-time PCR method as above, wherein two or more parts of the region of each variant are amplified, each part of the region comprising only one probe-binding site.

The real-time PCR method as above, wherein the different probes are labeled with different fluorescent reporter dyes.

The real-time PCR method as above, wherein the probes are labeled with FAM™ or VIC™.

The real-time PCR method as above for the detection and/or quantification of variants of the nucleic acid sequence of a virus.

The real-time PCR method as above for the detection and/or quantification of variants of the nucleic acid sequence of a retrovirus.

The real-time PCR method as above for the detection and/or quantification of variants of the nucleic acid sequence of a lentivirus.

The real-time PCR method as above for the detection and/or quantification of variants of the nucleic acid sequence of a Feline Immunodeficiency Virus (FIV).

The real-time PCR method as above, wherein the probes according to Seq. ID No. 3 and 6 are added to the reaction mixture.

The real-time PCR method as above, wherein the probes according to Seq. ID No. 3 and 9 are added to the reaction mixture.

The real-time PCR method as above, wherein the forward primer according to Seq. ID No. 1 and/or 12 and the reverse primer according to Seq. ID No. 2 are added to the reaction mixture.

The real-time PCR method as above, wherein the forward primer according to Seq. ID No. 4, 14, and/or 15 and the reverse primer according to Seq. ID No. 5 and/or 13 are added to the reaction mixture.

The real-time PCR method as above, wherein the forward primer according to Seq. ID No. 7, 20, and/or 21 and the reverse primer according to Seq. ID No. 8 are added to the reaction mixture.

The real-time PCR method as above, wherein the forward primer according to Seq. ID No. 16 and the reverse primer according to Seq. ID No. 17 or 19 are added to the reaction mixture.

The real-time PCR method as above, wherein the above listed primers and probes are added to the reaction mixture.

The real-time PCR method as above, wherein said PCR is a reverse-transcription (RT) PCR.

The real-time PCR method as above, wherein said variants of nucleic acid sequences are nucleic acid sequences derived from subtypes, isolates, clades or any other subgroup of a species.

Use of the real-time PCR method, as above, for the determination of the overall viral load in a sample comprising variants of a viral nucleic acid sequence.

The use of the real-time PCR method, as above, wherein the variants are derived from nucleic acid sequences derived from subtypes, isolates, clades or any other subgroup of a viral species.

The use of the real-time PCR method as above for the investigation of the impact of the viral load on tumorigenesis.

An oligonucleotide probe for use in a real-time PCR method selected from the group of probes comprising

(a) the nucleic acid sequences according to Seq. ID No. 3 and/or Seq. ID No. 6 and/or Seq. ID No. 9 and/or Seq. ID No. 18,

(b) their complementary strands, and/or

(c) nucleic acid sequences with a homology of at least about 70% to the nucleic acid sequences according to Seq. ID No. 3 and/or Seq. ID No. 6 and/or Seq. ID No. 9 and/or Seq. ID No. 18.

A primer for use in a PCR method selected from the group of primers comprising

(a) a primer according to Seq. ID No. 1, 2, 4, 5, 7 or 8 or 10 to 17 or 19 to 21,

(b) a primer complementary to one of said sequences, and/or

(c) a primer with a homology of at least about 70% to the nucleic acid sequences of one of said primers.

A set of primers selected from the group of primer sets comprising

(a) the primers according to Seq. ID No. 1 and/or 12 and according to Seq. ID No. 2,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID No. 4, 14, and/or 15 and according to Seq. ID No. 5 and/or 13,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID No. 7, 20, and/or 21 and according to Seq. ID No. 8,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID 16 and according to Seq. ID No. 17 and/or 19,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

A set of oligonucleotides for use in a real-time PCR method, comprising a primer set selected from the group of primer sets as above and a probe selected from the group of probes as above.

The set of oligonucleotides as above for use in the method as above.

Example: Detection of Proviral DNA

The following example will further illustrate the present invention. It will be well understood by a person skilled in the art that the provided example in no way may be interpreted in a way that limits the applicability of the technology provided by the present invention to this example.

Recently, a method based on the ABI 7700 system (Perkin Elmer, Foster City, California) was established and validated for the quantification of FIV proviral and viral loads (*Leutenegger et al. J Virol Methods 1999, 78(1):105-116*). In this method, the 5´nuclease activity of the Taq-polymerase allows the cleavage of a labeled probe and the subsequent liberation of a reporter fluorescent dye which can be excited with an argon laser and leads to the emission of light. The amount of emitted fluorescence, which is proportional to the amount of DNA produced during the PCR, is measured at regular intervals during the PCR and allows the monitoring of the PCR in a real-time manner. The method has been shown to be very useful in viral load determinations if just one isolate is used (e.g. in challenge experiments for vaccination trials where the isolate is known and optimized primers and probes could be used). However, as soon as viral loads from different isolates should be compared the equality of the PCR efficiency for the different isolates must be ensured. In the following example, the influence of mutations in the primer- and probe-binding site on the PCR-efficiency and the subsequent estimation of this influence on the viral load determination is analyzed. A real-time PCR system was established that allows the estimation of viral loads in patients, infected with different isolates as a basis for the determination of the impact of the viral load on tumorigenesis.

1 Conditions and parameters of the real-time PCR for the analysis of FIV sequences

First the conditions for the amplification of FIV sequences by real-time PCR were established and evaluated. Primers and probes for the amplification and detection of the gag gene of FIV were designed. The linear range within which the copy number of a template can be quantified was calculated using a plasmid with a corresponding

region of a FIV isolate. PCR-efficiencies were calculated for this plasmid and for a genomic standard.

1.1 Real-time PCR primers, probes, and cycling conditions

For the real-time PCR the subsequently described primer and probe sequences were designed using the Primer Express software (Perkin Elmer, Foster City, California). All oligonucleotides were purified by high-performance liquid chromatography and purchased from Perkin Elmer (Weiterstadt, Germany).

Three different real-time PCR assays (FIV1010p/v, FIV1416p, and FIV1372p) were developed. For the **FIV1010p**-assay, the PCR primers used were FIV0771f (5'-AGA ACC TGG TGA TAT ACC AGA GAC-3') and FIV1081r (5'-TTG GGT CAA GTG CTA CAT ATT G-3'). The primers were designed to be 100% homologous to the sequence of the clade A FIV isolates which comprise among other strains the isolates Petaluma (Genebank accession number M25381), San Diego PPR (M36968), Zurich1 (X57002), and Utrecht113 (X68019). It was also considered that for an efficient amplification the size of the amplified fragment should be smaller than 350 bp and, if possible, smaller than 100 bp.

For a monoplex PCR, the probe was labeled at the 5'end with the fluorochrome FAM (6-carboxy-fluorescein) which serves as a reporter fluorochrome and at the 3'end with the fluorochrome TAMRA (6-carboxy-tetramethyl-rhodamine) which functions as a quencher. In a multiplex PCR the same probe was labelled with the reporter fluorochrome VIC to distinguish between the signal of the different PCR systems.

The probe was designed, based on several criteria: (i) 8-10°C higher melting temperature than the primers, (ii) no G's at the 5' end of the probe, (iii) no stretches of identical nucleotides longer than four, especially not of G's, (iv) lack of self-annealing, (v) lack of predicted dimer formation with corresponding primers. Furthermore, the probe is blocked at the 3' end to prevent elongation during the amplification. The probe was at least 80%, but preferably 95 to 100% homologous to the sequence of different FIV isolates. The probe used to establish standard assay conditions was FIV1010p/v (5'-FAM/VIC-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3').

Under the same considerations as described for the FIV1010p-assay the two other assays were designed. The **FIV1416p**-assay was specific for the clade A FIV isolate Glasgow 8 (*Hosie, M.J., Jarrett, O. AIDS 1990, 4, 215-220*) and several FIV isolates from southern Germany or Austria. The system consisted of the primers FIV1360f (5'-GCA GAA GCA AGA TTT GCA CCA-3') and FIV1437r (5'-TAT GGC GGC CAA TTT TCC T-3'), and the probe FIV1416p (5'-FAM-TGC CTC AAG ATA CCA TGC TCT ACA CTG CA-TAMRA-3'). The **FIV1372p**-assay was designed to be 100% homologous to clade B FIV isolates Italy-M2 (Y13867), Italy-M3 (Y13866), Italy-M8 (Z96111), Amori-1 (D37823), Amori-2 (D37824), Sendai-2 (D37821), Yokohama (D37819), and a local, subtype B-like isolate. The system consisted of the primers FIV1280f (5'-ATC CTC CTG ATG GGC CTA GAC-3') and FIV1426r (5'-ACT TTC CTA ATG CTT CAA GGT ACC A-3') and the probe FIV1372p (5'-TTT GCA CCA GCC AGA ATG CAG TGT AG-3').

The target sequence was amplified in a 25µl reaction volume using the following PCR-conditions: 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 nM dATP, dCTP, dGTP, 400 nM dUTP, 300 nM of each primer, 200 nM of the fluorogenic probe, and 2.5 units of Taq DNA polymerase were used. After the initial

denaturation (2 min at 95°C), amplification was performed in 45 cycles each comprising 15 sec at 95°C and 60 sec at 60°C. For a multiplex PCR the same reaction conditions were applied.

1.2 Preparation of DNA

A Construct of the FIV Zurich 2 isolate (plasmid, pBSCompZ2 [Allenspach, et al., *Schweiz Arch Tierheilkd* 1996, *138,* 87-92]) was used as a control to determine the linear range of the real-time PCR. The plasmid was propagated in E. coli cells and extracted using the Qiagen Plasmid Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The copy number of the plasmid was estimated from the absorbtion at 260nm. A set of tenfold dilutions was performed in PCR grade water containing calf thymus DNA as a carrier in a concentration of 30 µg/ml.

A genomic DNA standard was developed that mimics the *in vivo* situation of a provirus integrated into genomic DNA of a cell. The DNA from CrFK cells [Crandell, et al., *In Vitro* 1973, *9,* 176-185] stably infected with different FIV isolates (Petaluma [Pedersen, et al., *Science* 1987, *235,* 790-793], Glasgow 8 [Hosie & Jarrett, *Aids* 1990, *4,* 215-220], Amsterdam 6 [Siebelink, et al., *Vet Immunol Immunopathol* 1995, *46,* 61-69], Utrecht 113 [Verschoor, et al., *J Clin Microbiol* 1993, *31,* 2350-2355] was extracted using the QIAamp Kit according to manufacturer's instructions (Qiagen, Hilden Germany). The DNA concentration was estimated by OD measurement at 260 nm and a tenfold dilution series was performed in PCR grade water containing 30 µg cellular DNA per ml.

When the proviral load was studied DNA was extracted from peripheral blood leucocytes. Otherwise, it was proceeded as described above for the DNA-extraction from infected CrFK cells.

1.3 Calculation of the template copy number

An algorithm of the Sequence Detection Software compares the amount of reporter dye emission (R) with the quenching dye emission (Q) once every few seconds during the PCR amplification, generating a normalized reporter signal ΔR_n. This value represents the fluorescence signal of the reporter dye divided by the fluorescence signal of the quencher signal minus the baseline signal established in the first few cycles of the PCR when cleaved probe is generally not detectable. The ΔR_n values are plotted as a function of the PCR cycle. The first cycle which is above a defined threshold (normally ten times the standard deviation of the background fluorescence) is defined as the threshold cycle C_T. Within a certain range of template concentrations the C_T value is proportional to the template copy number present at the beginning of the reaction and reflects the first opportunity for quantification of the template *(Heid, et al., 1996, Genome Research, 6, p. 986ff)*.

1.4 Accuracy of the quantification of control DNA

A dilution of the plasmid pBSCompZ2 was obtained as described above and the concentration was estimated in three independent real-time PCRs using the FIV1010p-assay for each of the diluted samples. The calculation of the initial copy number using ΔR_n is highly reproducible as it was shown by small standard deviations of the C_T values, ranging from 0.13 to 2.99%. With a decreasing number of

template copies the number of cycles increased and larger standard deviations are obtained. Hence, the accuracy of the measurement deteriorates.

Using the PCR conditions described above a linear relationship between C_T and the standard template concentration was achieved for nine log units (5×10^0 to 5×10^9 copies). The coefficient of correlation, that is defined as the percentage of standard deviation of the threshold cycle numbers, was 0.9977. This high coefficient of correlation is a prerequisite for the calculation of the PCR-efficiency. The result confirms that the method is highly accurate over a wide range of template concentrations.

2.4 Calculation of the PCR-efficiency

The PCR-efficiency can be used to evaluate the PCR-conditions. If the PCR-efficiency was 100% the concentration of the target sequence should be doubled every cycle. However, usually the PCR-efficiency (**E**) is less than 100% and the amount of PCR-product (**Y**) amplified from an initial template copy number **Z** after **n** cycles can be calculated according to the following equation $\mathbf{Y=Z \times (1+E)^n}$ or after logarithmic transformation as $\mathbf{\log Y = \log Z + n \times \log (1 + E)}$. The PCR-efficiency **E** can be calculated from the slope of a standard curve where the C_T-value is plotted against the logarithm of the copy number of a dilution serie. Now the PCR-efficiency can be described as $\mathbf{E= 10^{-1/s} -1}$ with s representing the slope of the straight line.

A set of tenfold dilutions of the FIV plasmid was prepared as described above. A real-time PCR using the FIV1010p-assay was performed for each dilution and a standard curve was obtained. A PCR-efficiency of 0.9815 was calculated from the slope of that standard curve.

In addition, the PCR-efficiency of the genomic standard obtained from transduced CrFK cells as described above was calculated. A real-time PCR was performed and PCR-efficiencies were calculated. The PCR-efficiencies varied between 0.9742 and 0.8711. The best PCR-efficiency (0.9742 for CrFK cells infected with FIV Petaluma) is almost as good as the PCR-efficiency of the plasmid dilution series.

For all constructs the correlation coefficient was larger than 0.99 which demonstrates that a comparison of the PCR-efficiencies is possible. The results show that the chosen PCR conditions are suitable for an efficient amplification of the FIV fragment.

2.5 Influence of mismatches on the PCR-efficiency

The viral load can only be compared between different samples if the PCR-efficiencies for the different samples are similar, i.e. when the reaction conditions are suitable for the amplification of a specific template. In vaccination trails where an organism is challenged with a specific viral subtype the PCR conditions can be optimised for this specific viral subtype allowing the comparison of data between patients infected with the same isolate. The same accuracy needs to be achieved in naturally infected patients, where a swarm of viruses differing in their nucleotide sequence can be present. The FIV model was chosen in order to investigate the influence of mismatches on the PCR-efficiency.

2.5.1 Sequence analysis of a conserved region of the FIV genome

The complete *gag* gene (1.6 kb) of the characterised isolates of FIV (Petaluma, Glasgow 8, Amsterdam 6 and Utrecht 113) were amplified and sequenced using the

primers FIV566f (5'-ACC TTC AAG CCA GGA GAT TC-3') and FIV2167r (5'-CCT CCT CCT ACT CCA ATC AT-3'). Additionally, a 311 bp region of the FIV*gag* gene of some of the unclassified isolates (Munich 3, 4, 6 and 7) was amplified and sequenced with the same primers as for the FIV1010p-assay. The used primers were FIV0771f (5'-AGA ACC TGG TGA TAT ACC AGA GAC-3') and FIV1081r (5'-TTG GGT CAA GTG CTA CAT ATT G-3').

A conventional PCR was performed on a 9600 thermal cycler (Perkin Elmer, Foster City, California). PCR reactions contained 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 200 nM dATP, dCTP, dGTP, dTTP, 300 nM of each primer and 2.5 U of Taq DNA polymerase. Amplification was performed with 1 cycle of 3 min at 95°C, 60 sec at 51°C and 3 min at 72°C, followed by 39 cycles of 15 sec at 94°C, 40 sec at 51°C and 90 sec at 72°C. PCR-products were separated on a 0.8 % agarose gel and visualized after ethidium bromide staining with the Eagle Eye system (Stratagene, Heidelberg, Germany). The appropriate bands were isolated and DNA was purified using the QIAamp gel extraction Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Approximately 20 - 50 ng PCR-product were used in the subsequent sequencing reaction mixture containing 4 µl BigDye premix (Perkin Elmer, Foster City, California), 4 pmol of the primer FIV0771f, and water in a total volume of 10 µl. The cycling was performed on a 9600 thermocycler (Perkin Elmer, Foster City, California) with the following programme: 30 sec at 96°C, 10 sec at 50°C, 4 min at 60°C for 30 cycles. The sequencing reaction was purified according to the manufacturer's instructions. The sequence analysis was performed with an ABI 310 Genetic Analyzer (Perkin Elmer, Foster City, California).

2.5.2 Comparison of sequence data and real-time PCR-efficiency for standard isolates

Four isolates were sequenced and, in parallel, amplified by real-time PCR using the FIV1010p-assay as described above. The sequence data and the corresponding PCR-efficiencies are listed in Table 1. In the region of the forward primer (FIV0771f) no mutation was found in any of the four isolates. In the region where the probe FIV1010p anneals only one mutation was found. The T to C change at bp 1020 was found when the sequence of the Amsterdam 6 isolate [Siebelink, et al., *Vet Immunol Immunopathol* 1995, *46,* 61-69] was compared with the sequence of FIV Petaluma (Genebank accession number M25381). Interestingly, the nucleotide sequence of this isolate was still detected but with the lowest PCR-efficiency (0,8711) compared to all other isolates. In the region of the reverse primer FIV1081r one point mutation was found. In the Glasgow 8 isolate [Hosie, & Jarrett, *Aids* 1990, *4,* 215-220] an A to T change at position 1071 was detected, which was associated with the second lowest PCR-efficiency of 0.9284. In summary, in the four standard isolates two point mutations were found, which reduced the PCR-efficiency.

2.5.3 Comparison of sequence data and PCR-efficiencies for field isolates

The real-time PCR system was analysed for FIV isolates of ten naturally infected cats. The cats were selected from southern Germany and Austria. This region has previously been shown to contain a heterogenous FIV population. In that region isolates from three different subtypes and from several other genetic outliers have been found [Bachmann, M. H., et al., *J Virol* 1997, *71, pp.* 4241ff].

Five out of ten tested cats infected with unknown FIV isolates were positive in the real-time PCR assay (Table 2). However, two of the five cats (Munich 3 and 4) that were negative in the real-time PCR, were positive when the PCR-product was analysed by agarose gel electrophoresis. Sequencing showed that the sequence of the two FIV isolates Munich 3 and 4 differed from the previously published sequences

by three and four mutations in the probe-binding site respectively. The mismatches are located in a part of the probe which is initially not displaced by the Taq polymerase and which is responsible for the binding of the probe before the probe is cleaved by the 5' nuclease activity of the Taq-polymerase. The failed amplification can either be explained by the lack of binding or by the displacement of the probe before appropriate cleavage.

In contrast, no mutations were found in the probe-binding site of the two isolates, which were positive in the conventional and in the real-time PCR (Table 2). For these two samples the curve of the real-time PCR showed an exponential increase of the fluorescence signal similar to the one which was seen for the plasmid standard indicating a high PCR-efficiency. In conclusion, variation in the probe-binding site result in apparently reduced PCR-efficiency or even PCR-failure.

6 Detection of different FIV clades by multiplex real-time PCR

The proviral load was studied according to the invention for samples of cats infected with FIV. It was shown that using more than one primer pair and probe allows the detection of a larger number of viral strains and to differentiate between subtypes.

FIV isolates of an unknown subtype were analysed using the above described conditions. Three monoplex real-time PCRs were performed: The assays FIV1010v and FIV1416p that are specific for clade A FIV isolates and the FIV1372p-assay that is specific for the clade B subtype. Two multiplex PCRs were performed: The FIV1010v- and the FIV1416p- assay were used in a multiplex real-time PCR to detect clade A FIV isolates. The FIV1010v- and the FIV1372p-assay were used to detect clade A and clade B FIV isolates in a multiplex setup. The use of different

reporter dyes (VIC™ in the FIV1010v-assay and FAM™ in the FIV1372p and in the FIV1416p-assay) allowed to distinguish between the signal of the two PCR-systems in the multiplex setup.

The results of the FIV1010p, FIV 1416p or the FIV1372p monoplex real-time PCR are compared with the multiplex real-time PCR FIV1010v/FIV1416p and FIV1010v/FIV1372p. The results of the FIV1010p and FIV1372p assay are summarized in Table 3. For more than one third of the samples that were analyzed by one monoplex real-time PCR no signal was detected. Surprisingly, combining the FIV1010v and the FIV1372p assay none of 30 samples was negative. Hence, the use of more than one primer pair and more than one probe enabled the detection of all samples which were not detected by one monoplex real-time PCR.

The inventors used the results to group the viruses of the different samples into clades. In 6 out off 30 samples the results of two monoplex real-time PCRs were inconclusive. In contrast, the results of one multiplex real-time PCR allowed the grouping into clade A or B for all samples. In conclusion, the present invention allows to detect sequences of different viral clades and also to group the viruses according to their sequence into the different clades.

The final goal however will be a system that allows the detection and quantification of a broad range of different isolates with similar PCR efficiencies. Based on this optimized system the comparison of viral loads from cats infected with different FIV isolates can be performed much more precisely. Such an optimized system provides the tool for the investigation of the impact of the viral load on the development of cancers in lentiviral infection, as well as providing the basis for the investigation of the efficiency of therapeutic agents tested in naturally infected cats. Furthermore, real-time PCR strategies can be designed to detect mutations in oncogenes present in

biopsy material, where tumor and normal cells may be present. In such cases, real-time PCR permits a quantification of the number of tumor cells present.

Table 1: Comparison of PCR-efficiencies of the FIV1010p-assay and sequence variation in the oligonucleotide binding site.

Origin of the sequence	PCR-efficiency	Sequence		
		FIV0771f	FIV1010p	FIV1081r
Oligonucl.		agaacctggtgatataccagagac	aggagggccctccacaggcata	caatatgtagcacttgacccaa
Petaluma	0.9742	----------	----------	----------
Glasgow8	0.9284	----------	----------	----------t----------
Amsterdam6	0.8711	----------	---------c----------	----------
Utrecht113	0.9485	----------	----------	----------

Table 2: Comparison of the real-time PCR results and sequence variation in the probe-binding site.

Field isolate	Agarose gel electrophoresis	Real-Time PCR	FIV1010P binding site
Oligonucl.			agg agg gcc ctc cac agg cat a
Munich 3	+	-	-a- --- -a- --- --- --- -t- -
Munich 4	+	-	-g- --a aa- --- --- --- --- -
Munich 5	-	-	nd
Munich 6	+	+	--- --- --- --- --- --- --- -
Munich 7	+	+	--- --- --- --- --- --- --- -
Munich 1	+	+	nd
Munich 2	+	+	nd
Munich 8	-	-	nd
Munich 9	+	+	nd
Munich 10	-	-	nd

Table 3: Amplification of 25 unknown FIV isolates by three monoplex or two multiplex real-time PCRs

	PCR-assay			
FIV isolate	1010p	1372p	1010v/1372p	subtype
Munich 11	+	-	+/-	A
Munich 14	-	+	-/+	B
Munich 18	-	+	-/+	B
Munich 20	-	+	-/+	B
Munich 27	-	+	-/+	B
Munich 29	-	+	-/+	B
Munich 31	+	-	+/-	A
Munich 32	+	-	+/-	A
Munich 35	+	-	+/-	A
Munich 36	+	-	+/-	A
Munich 38	-	+	-/+	B
Munich 39	-	+	-/+	B
Munich 40	-	+	-/+	B
Munich 41	+	-	+/-	A
Munich 43	-	+	-/+	B
Munich 44	+	-	+/-	A
Munich 49	-	+	-/+	B
Munich 50	-	+	-/+	B
Munich 52	+	-	+/-	A
Munich 53	+	-	+/-	A
Utrecht 113	+	-	+/-	A
Petaluma	+	-	+/-	A
Amsterdam 6	+	-	+/-	A
Italy M2*	+	+	-/+	B
Italy M20*	+	+	-/+	B
Austria 01*	+	+	-/+	B
Austria 02	+	-	+/-	A
Austria TE*	+	+	-/+	B
Austria 05*	+	+	-/+	B
Austria 06*	+	+	-/+	B

Claims

1. A real-time Polymerase Chain Reaction (PCR) method for the detection and/or quantification of variants of a nucleic acid sequence, wherein the same region of said variants is completely or partially to be amplified, each variant differing in one or more nucleotides within the probe-binding site, said method comprising addition of two or more oligonucleotide probes to the same PCR mixture, each probe being specific for the probe binding site of at least one variant.

2. The real-time PCR method according to claim 1, wherein said variants of the nucleic acid sequence differ in one or more nucleotides within the primer binding sites and wherein more than one primer pair is added to the reaction mixture each primer specifically annealing to the primer binding site of at least one subtype

3. The real-time PCR method according to claim 1 or 2, wherein two or more parts of the region are amplified, each part of the region comprising only one probe binding site.

4. The real-time PCR method according to any of the preceding claims 1 to 3, wherein the different probes are labeled with different fluorescent reporter dyes.

5. The real-time PCR method according to claim 4, wherein the probes are labeled with FAM™ or VIC™.

6. The real-time PCR method according to any of the preceding claims 1 to 5 for the detection and/or quantification of variants of the nucleic acid sequence of a virus.

7. The real-time PCR method according to claim 6 for the detection and/or quantification of variants of the nucleic acid sequence of a retrovirus.

8. The real-time PCR method according to claim 7 for the detection and/or quantification of variants of the nucleic acid sequence of a lentivirus.

9. The real-time PCR method according to claim 8 for the detection and/or quantification of variants of the nucleic acid sequence of a Feline Immunodeficiency Virus (FIV).

10. The real-time PCR method according to claim 9, wherein the probes according to Seq. ID No. 3 and 6 are added to the reaction mixture.

11. The real-time PCR method according to claim 9, wherein the probes according to Seq. ID No. 3 and 9 are added to the reaction mixture.

12. The real-time PCR method according to claim 9, wherein the forward primer according to Seq. ID No. 1 and/or 12 and the reverse primer according to Seq. ID No. 2 are added to the reaction mixture.

13. The real-time PCR method according to claim 9, wherein the forward primer according to Seq. ID No. 4, 14, and/or 15 and the reverse primer according to Seq. ID No. 5 and/or 13 are added to the reaction mixture.

14. The real-time PCR method according to claim 9, wherein the forward primer according to Seq. ID No. 7, 20, and/or 21 and the reverse primer according to Seq. ID No. 8 are added to the reaction mixture.

15. The real-time PCR method according to claim 9, wherein the forward primer according to Seq. ID No. 16 and the reverse primer according to Seq. ID No. 17 and/or 19 are added to the reaction mixture.

16. The real-time PCR method according to claim 9, wherein the primers according to claim 12 and/or 13 and the probes according to claim 10 are added to the reaction mixture.

17. The real-time PCR method according to claim 9, wherein the primers according to claim 12 and/or 14 and the probes according to claim 11 are added to the reaction mixture.

18. The real-time PCR method according to any of the preceding claims 1 to 17, wherein said PCR is a reverse-transcription (RT) PCR.

19. The real-time PCR method according to any of the preceding claims 1 to 18, wherein said variants of nucleic acid sequences are nucleic acid sequences derived from subtypes, isolates, clades or any other subgroup of a species.

20. Use of the real-time PCR method according to any of the preceding claims 6 to 19 for the determination of the overall viral load in a sample comprising variants of a viral nucleic acid sequence.

21. The use of the real-time PCR method according to claim 20, wherein the variants are derived from nucleic acid sequences derived from subtypes, isolates, clades or any other subgroup of a viral species.

22. The use of the real-time PCR method according to claims 20 or 21 for the investigation of the impact of the viral load on tumorigenesis.

23. An oligonucleotide probe for use in a real-time PCR method selected from the group of probes comprising

(a) the nucleic acid sequences according to Seq. ID No. 3 and/or Seq. ID No. 6 and/or Seq. ID No. 9 and/or Seq. ID No. 18,

(b) their complementary strands, and/or

(c) nucleic acid sequences with a homology of at least about 70% to the nucleic acid sequences according to Seq. ID No. 3 and/or Seq. ID No. 6 and/or Seq. ID No. 9 and/or Seq. ID No. 18.

24. A primer for use in a PCR method selected from the group of primers comprising

(a) a primer according to Seq. ID No. 1, 2, 4, 5, 7 or 8 or 10 to 17 or 19 to 21,

(b) a primer complementary to one of said sequences, and/or

(c) a primer with a homology of at least about 70% to the nucleic acid sequences of one of said primers.

25. A set of primers selected from the group of primer sets comprising

(a) the primers according to Seq. ID No. 1 and/or 12 and according to Seq. ID No. 2,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

26. A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID No. 4, 14, and/or 15 and according to Seq. ID No. 5 and/or 13,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

27. A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID No. 7, 20, and/or 21 and according to Seq. ID No. 8,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

28. A set of primers selected from the group of sets of primers comprising

(a) the primers according to Seq. ID 16 and according to Seq. ID No. 17 and/or 19,

(b) primers with a nucleic acid sequence complementary to one or more of the primers according to (a), and/or

(c) a primer with a nucleic acid sequence with a homology of at least about 70% to the primers according to (a).

29. A set of oligonucleotides for use in a real-time PCR method, comprising a primer set selected from the group of primer sets according to any of the preceding claims 25 to 28 and a probe selected from the group of probes according to claim 23.

30. The set of oligonucleotides according to claim 29 for use in the method according to claim 9.

SEQUENCE LISTING

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<120> Multiplex Real-Time PCR

<130> Sequence listing for BN29PCT

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